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Effect modification by population dietary folate on the association between MTHFR genotype, homocysteine, and stroke risk: a meta-analysis of genetic studies and randomised trials

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Abstract: **BACKGROUND:** The MTHFR 677C→T polymorphism has been associated with raised homocysteine concentration and increased risk of stroke. A previous overview showed that the effects were greatest in regions with low dietary folate consumption, but differentiation between the effect of folate and small-study bias was difficult. A meta-analysis of randomised trials of homocysteine-lowering interventions showed no reduction in coronary heart disease events or stroke, but the trials were generally set in populations with high folate consumption. We aimed to reduce the effect of small-study bias and investigate whether folate status modifies the association between MTHFR 677C→T and stroke in a genetic analysis and meta-analysis of randomised controlled trials. **METHODS:** We established a collaboration of genetic studies consisting of 237 datasets including 59,995 individuals with data for homocysteine and 20,885 stroke events. We compared the genetic findings with a meta-analysis of 13 randomised trials of homocysteine-lowering treatments and stroke risk (45,549 individuals, 2314 stroke events, 269 transient ischaemic attacks). **FINDINGS:** The effect of the MTHFR 677C→T variant on homocysteine concentration was larger in low folate regions (Asia; difference between individuals with TT versus CC genotype, $3 \cdot 12$ mol/L, 95% CI $2 \cdot 23$ to $4 \cdot 01$) than in areas with folate fortification (America, Australia, and New Zealand, high; $0 \cdot 13$ mol/L, $-0 \cdot 85$ to $1 \cdot 11$). The odds ratio (OR) for stroke was also higher in Asia ($1 \cdot 68$, 95% CI $1 \cdot 44$ to $1 \cdot 97$) than in America, Australia, and New Zealand, high ($1 \cdot 03$, $0 \cdot 84$ to $1 \cdot 25$). Most randomised trials took place in regions with high or increasing population folate concentrations. The summary relative risk (RR) of stroke in trials of homocysteine-lowering interventions ($0 \cdot 94$, 95% CI $0 \cdot 85$ to $1 \cdot 04$) was similar to that predicted for the same extent of homocysteine reduction in large genetic studies in populations with similar folate status (predicted RR $1 \cdot 00$, 95% CI $0 \cdot 90$ to $1 \cdot 11$). Although the predicted effect of homocysteine reduction from large genetic studies in low folate regions (Asia) was larger (RR $0 \cdot 78$, 95% CI $0 \cdot 68$ to $0 \cdot 90$), no trial has evaluated the effect of lowering of homocysteine on stroke risk exclusively in a low folate region. **INTERPRETATION:** In regions with increasing levels or established policies of population folate supplementation, evidence from genetic studies and randomised trials is concordant in suggesting an absence of benefit from lowering of homocysteine for prevention of stroke. Further large-scale genetic studies of the association between MTHFR 677C→T and stroke in low folate settings are needed to distinguish effect modification by folate from small-study bias. If future randomised trials of homocysteine-lowering interventions for stroke prevention are undertaken, they should take place in regions with low folate consumption.

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Effect modification by population dietary folate on the association between *MTHFR* genotype, homocysteine, and stroke risk: a meta-analysis of genetic studies and randomised trials

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Summary

Background—The *MTHFR* 677C→T polymorphism has been associated with raised homocysteine concentration and increased risk of stroke. A previous overview showed that the effects were greatest in regions with low dietary folate consumption, but differentiation between the effect of folate and small-study bias was difficult. A meta-analysis of randomised trials of homocysteine-lowering interventions showed no reduction in coronary heart disease events or stroke, but the trials were generally set in populations with high folate consumption. We aimed to reduce the effect of small-study bias and investigate whether folate status modifies the association between *MTHFR* 677C→T and stroke in a genetic analysis and meta-analysis of randomised controlled trials.

Methods—We established a collaboration of genetic studies consisting of 237 datasets including 59 995 individuals with data for homocysteine and 20 885 stroke events. We compared the genetic findings with a meta-analysis of 13 randomised trials of homocysteine-lowering treatments and stroke risk (45 549 individuals, 2314 stroke events, 269 transient ischaemic attacks).

Findings—The effect of the *MTHFR* 677C→T variant on homocysteine concentration was larger in low folate regions (Asia; difference between individuals with TT versus CC genotype, 3.12 µmol/L, 95% CI 2.23 to 4.01) than in areas with folate fortification (America, Australia, and New Zealand, high; 0.13 µmol/L, −0.85 to 1.11). The odds ratio (OR) for stroke was also higher in Asia (1.68, 95% CI 1.44 to 1.97) than in America, Australia, and New Zealand, high (1.03, 0.84 to 1.25). Most randomised trials took place in regions with high or increasing population folate concentrations. The summary relative risk (RR) of stroke in trials of homocysteine-lowering interventions (0.94, 95% CI 0.85 to 1.04) was similar to that predicted for the same extent of homocysteine reduction in large genetic studies in populations with similar folate status (predicted RR 1.00, 95% CI 0.90 to 1.11). Although the predicted effect of homocysteine reduction from large genetic studies in low folate regions (Asia) was larger (RR 0.78, 95% CI 0.68 to 0.90), no trial has evaluated the effect of lowering of homocysteine on stroke risk exclusively in a low folate region.

Interpretation—In regions with increasing levels or established policies of population folate supplementation, evidence from genetic studies and randomised trials is concordant in suggesting an absence of benefit from lowering of homocysteine for prevention of stroke. Further large-scale genetic studies of the association between *MTHFR* 677C→T and stroke in low folate settings are needed to distinguish effect modification by folate from small-study bias. If future randomised trials of homocysteine-lowering interventions for stroke prevention are undertaken, they should take place in regions with low folate consumption.

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Introduction

Prospective cohort studies previously estimated that, if causal, a reduction of 3 $\mu\text{mol/L}$ in serum homocysteine would decrease risk of coronary heart disease by 18% and stroke by 24% after a mean follow-up of 7.3 years. However, residual confounding and reverse causation are alternative non-causal explanations for the observational association. Mendelian randomisation studies use genetic variants as proxies of non-genetic risk factors to assess whether a risk factor is causally related to a disease. The approach exploits the independent randomised assortment of maternal and paternal alleles at the time of gamete formation to reduce confounding, and the fixed nature of genotype to abolish reverse causality and minimise regression-dilution bias, which can affect the association of a non-genetic risk factor with disease. Mendelian randomisation analyses have previously investigated the potential causal role of homocysteine in stroke and coronary heart disease using the *MTHFR* 677C→T variant (rs1801133) as a proxy for homocysteine concentration that is independent of other risk factors, and have provided support for a causal role of homocysteine in stroke. However, literature-based genetic meta-analyses can be affected by small-study bias. For instance, in our previous mendelian randomisation analysis of *MTHFR* 677C→T, only two studies had more than 400 stroke cases.

In addition to our original study, results of other studies also suggest that the effect of the *MTHFR* 677C→T variant on homocysteine concentration could be modified by the prevailing concentrations of folic acid, with high folate being associated with a reduced effect of *MTHFR* 677C→T on homocysteine concentrations. This suggestion is further supported by evidence that low folate intake or concentrations are associated with increased stroke risk. This gene–environment interaction is biologically plausible because folic acid plays a central part in the metabolism of homocysteine. Similarly, effect modification of folic acid on the *MTHFR* 677C→T effect has also been suggested for colon cancer. If prevailing concentrations of folic acid do modify homocysteine concentration, established policies for folic acid fortification of cereals and flour in several countries, for the prevention of neural tube defects, would also be expected to modify the association between *MTHFR* 677C→T and stroke risk. This effect would also modify the interpretation of published randomised clinical trials of homocysteine-lowering interventions for stroke prevention that have shown no reduction in stroke risk in meta-analysis, but trials to date have been predominantly set in populations with high folate consumption.

To investigate the potential modifying effect of folate status on the association between the *MTHFR* 677C→T variant and stroke risk, we established a collaboration of genetic studies including data for homocysteine concentration and stroke events. We compared the findings of this updated genetic analysis with a meta-analysis of randomised controlled trials of homocysteine-lowering treatments on stroke risk.

Methods

Search strategy and selection criteria

Webappendix pp 1–7 provides detailed information about methods used. We did a literature search in Medline up to August, 2010, to identify randomised controlled trials using homocysteine-lowering interventions that evaluated cardiovascular endpoints. Medline and Embase were searched up to January, 2008, for all studies of the association between the rs1801133 polymorphism (*MTHFR* 677C→T variant) and stroke. To minimise reporting and publication bias, we supplemented our database with unpublished data by contacting investigators who reported genetic findings in stroke or cardiovascular disease in peer-reviewed journals.

Data extraction

Data were extracted and entered into a database for randomised trials (MVH and JPC) and genetic studies (PN, MVH). Genetic studies were classified into five categories of probable folate status. Full details of this process (developed and validated by Robert Clarke, Clinical Trial Service Unit and Epidemiological Studies Unit, University of Oxford, UK) are provided on webappendix pp 1–7. In brief, Robert Clarke undertook a systematic review of population-based studies in adults that measured folic acid concentration or quantified folate intake from nutritional questionnaires. Information about geographical location of study, years of conduct, and regional folic acid fortification policies (including years in which policies were established, if relevant) were used to generate five categories approximating the probable folate status at the population level. Categories were sorted from lowest to highest probable folate status: (1) no fortification (Asia, north and sub-Saharan Africa); (2) low (prefortification in 1996: Europe, including Ireland, Scandinavia, the Netherlands, Russia, and Turkey); (3) mid (postfortification in 1996: Europe); (4) mid (prefortification: America, Australia, and New Zealand before 1996; Central and South America); (5) high postfortification (America, Australia, and New Zealand after 1996, Chile after 2000).

Statistical analysis

Statistical analysis followed guidelines from the HuGE Review Handbook for meta-analysis of genetic association studies and the Cochrane Handbook for Systematic Reviews of Interventions. In all meta-analyses, the presence of small-study bias was investigated by funnel plot and Egger test. Additionally, we attempted to quantify the potential effect of small-study bias by restricting the analysis to large studies only, using the trim-and-fill method, and estimating the number of null hypothetical studies that were needed to add to change the summary effect from the meta-analysis (see webappendix pp 1–7 for further details).

Random effect models (DerSimonian and Laird) were used to obtain the mean difference in concentrations of homocysteine according to *MTHFR* 677C→T genotype (CC, homozygous common allele; CT, heterozygous; and TT, homozygous rare allele). Individuals homozygous for the C allele were used as the reference group. We did an ad-hoc analysis to investigate whether differences in plasma folate by the *MTHFR* 677C→T variant were also modified by folate status category.

We did a meta-analysis of published and unpublished genetic studies on stroke to obtain a per-genotype summary odds ratio (OR) and corresponding 95% CI (using random effect models, DerSimonian and Laird) for all stroke types combined, using individuals homozygous for the C allele as the reference group.

To check for confounding, we used Genome-wide Linkage Disequilibrium Repository and Search engine (GLIDERS) software to evaluate long-range linkage disequilibrium for the rs1801133 variant in different ethnic groups. We crosschecked retrieved single nucleotide polymorphisms (SNPs) in genome-wide association study repositories (NHGRI GWAS Catalog and SNPnexus) to confirm or refute whether any of these variants were associated with stroke or a stroke-related trait (eg, blood pressure).

Finally, we did a meta-analysis of randomised trials to estimate the effect of homocysteine-lowering treatment (ie, folic acid supplementation with or without additional B vitamins) on stroke risk in trials using placebo, low dose-folic acid, or usual care as the comparator group. We used the DerSimonian and Laird Q test in all meta-analyses to evaluate the degree of heterogeneity between studies, and the I^2 measure to describe the proportion of total variation in study estimates attributable to heterogeneity.

Role of the funding source

The funding sources had no role in study design, in the collection, analysis, and interpretation of data, in writing of the report, or in the decision to submit for publication. The corresponding author (JPC) had full access to all data in the study and had final responsibility for the decision to submit for publication.

Results

We established a collaboration of genetic studies consisting of 237 datasets, including 59 995 individuals with data for homocysteine and 20 885 stroke events. For analysis of homocysteine and the *MTHFR* 677C→T polymorphism, 98 datasets from 79 studies, including 59 995 individuals, met our selection criteria. 67 datasets (webappendix pp 18–19) including 53 643 participants without evidence of cardiovascular disease contributed to the analysis of the association between *MTHFR* 677C→T and homocysteine concentration. A histogram of sample sizes (webappendix p 8) and genotype frequencies according to folate status category and by ethnic group (webappendix p 9–10) are provided. Individuals from the remaining 31 datasets (of 98) were excluded because they had cardiovascular disease, which could affect the derived value of the gene variant on homocysteine concentrations. In individuals without cardiovascular disease, irrespective of probable folate status, the mean difference in homocysteine concentrations between those homozygous for the T allele compared with those homozygous for the C allele was 2.10 $\mu\text{mol/L}$ (95% CI 1.71 to 2.50; $I^2=83.5\%$). For heterozygous individuals, the mean difference in homocysteine concentration was 0.42 $\mu\text{mol/L}$ (95% CI 0.30 to 0.54; $I^2=49.3\%$) compared with those with the CC genotype.

The *MTHFR* 677C→T effect on homocysteine was highly dependent on probable folate status category (figure 1). In regions with no supplementation with folic acid (Asia), participants homozygous for the T allele had higher concentrations of homocysteine (3.12 $\mu\text{mol/L}$, 95% CI 2.23 to 4.01) than did those homozygous for the C allele. The effect was reduced in studies undertaken in geographical regions with policies of folic acid fortification (America, Australia, and New Zealand, high: 0.13 $\mu\text{mol/L}$, 95% CI –0.85 to 1.11). When analysis was limited to large studies (≥ 500 individuals), similar results were obtained (figure 1). Patterns were similar when heterozygous individuals were compared with those homozygous for the C allele, although the magnitude of the genetic effect was smaller (figure 1).

A metaregression analysis showed an inverse association between difference in homocysteine concentration by genotype (TT vs CC) and probable folate status category coded in ascending order of probable levels of folic acid status (Asia; Europe, low; Europe,

mid; America, Australia, and New Zealand, mid; and America, Australia, and New Zealand, high), suggesting an interaction between *MTHFR* 677C→T variant and folate status. For a one-category increase in folate status, the genetic effect on homocysteine concentration was reduced by 0.62 µmol/L (95% CI 0.29 to 0.94). To explore possible effects of small-study bias, metaregression was restricted to only large studies (≥500 individuals) and a similar pattern was noted (β coefficient for a one-category increase was equal to a reduction in homocysteine of 0.54 µmol/L, 95% CI 0.05 to 1.03). In a subset of 35 studies totalling 12 007 participants with information about plasma folic acid concentration, metaregression analysis suggested that for every increase of 1 ng/mL in plasma folic acid concentration, the mean difference in homocysteine concentrations for TT individuals versus CC individuals decreased by 0.13 µmol/L (95% CI −0.01 to 0.28). The Egger regression test (which tests the null hypothesis that the funnel plot is symmetrical) coefficient was 0.79 (95% CI −0.10 to 1.69; *p*=0.08) for TT versus CC comparison, and 0.36 (95% CI −0.14 to 0.87; *p*=0.16) for the CT versus CC comparison (including all studies, irrespective of geographical location). By contrast with a clear effect of folate status category on the *MTHFR*–homocysteine association, the evidence from our subsample of studies with data for plasma folic acid concentration did not reveal a clear trend between *MTHFR* and plasma folic acid across probable folate categories (data not shown).

140 datasets from 101 studies including 20 885 stroke events met our selection criteria and were included in the meta-analysis of *MTHFR* 677C→T polymorphism and stroke (webappendix pp 20–21). 94 datasets measured ischaemic stroke (17 909 cases), 20 haemorrhagic stroke (1615 cases), one silent brain infarction (161 cases), and 25 total stroke (6972 cases, including ischaemic and haemorrhagic cases, resulting in overlap between categories). Total stroke included studies that reported both haemorrhagic and ischaemic stroke and those that did not classify stroke because neuroimaging was unavailable. Of the studies that did not classify stroke, 17 were in individuals of European ancestry in whom most strokes are of ischaemic cause. Genotype frequencies according to folate status category and by ethnic origin are reported on webappendix p 11–12.

The summary OR for the main stroke comparison irrespective of probable folate status category was 1.37 (95% CI 1.25 to 1.50; *I*²=48.4%) for participants homozygous for the T allele compared with those homozygous for the C allele (webappendix p 13). The OR for heterozygous individuals was 1.14 (95% CI 1.08 to 1.21; *I*²=43.4%). When restricted to large studies (≥400 stroke events), the OR was 1.09 (95% CI 0.98 to 1.20; *I*²=24.6%) for TT individuals and 1.06 (95% CI 1.00 to 1.12; *I*²=0.0%) for CT individuals compared with those homozygous for the C allele. Results for different stroke subtypes followed a similar pattern (webappendix p 13).

In regions without folic acid fortification (Asia), the odds of stroke was 1.68 (95% CI 1.44 to 1.97) for comparison of individuals with the TT genotype and those with the CC genotype (figure 2). This increase in risk was substantially larger than that noted in regions with low folate intake (Europe, low: OR 1.01, 95% CI 0.88 to 1.16) and regions with mid folate intake (Europe, mid: OR 1.33, 95% CI 1.14 to 1.55) or folate fortification (America, Australia, and New Zealand, high: OR 1.03, 95% CI 0.84 to 1.25). A similar pattern was reported in comparisons of heterozygous (CT) individuals and those with the CC genotype (figure 2).

To examine the effect of small-study bias on the association between *MTHFR* TT versus CC in Asia and risk of stroke, we first restricted our analysis to studies with 400 or more stroke events, which yielded an OR of 1.28 (95% CI 1.11 to 1.48; figure 3). We then did a trim-and-fill analysis that, after taking into consideration the number and outcomes of potentially missing data, provided an adjusted summary OR equal to 1.30 (95% CI 1.19 to 1.43;

webappendix p 14), which was concordant with the point estimate derived from our analysis restricted to large studies. Last, we undertook a sensitivity analysis to estimate the effect on the point estimate of the OR for stroke risk in Asian studies (for the *MTHFR* TT vs CC comparison) from the sequential addition of hypothetical large and null studies of 400 stroke cases and 400 controls with equal distribution according to expected *MTHFR* C/T genotype frequencies in Asian ancestry (webappendix pp 4–5). We estimated that 41 such studies would be needed to reduce the overall point estimate from OR 1.68 to 1.15 (the threshold at which cumulative evidence in genetic studies would be downgraded on the basis of Venice criteria) and 260 such studies to reduce the lower bound of the 95% CI to 1.00; other hypothetical scenarios are reported on webappendix p 22. Furthermore, genetic studies of *MTHFR* and stroke from Asia contributed as many stroke cases as did other geographical regions, with no major differences in the mean allele frequency for the *MTHFR* C/T variant (webappendix pp 9–12).

A metaregression analysis showed that for a shift from one folate status category to the next highest category, the OR for stroke decreased by 10% (95% CI 1 to 18) for the TT versus CC comparison and 6% (95% CI 1 to 12) for the CT versus CC comparison. In a subsample of 25 studies (6266 stroke events) with information about both stroke and homocysteine concentration, the OR for the TT versus CC genotype tended to increase as the difference in homocysteine concentration by genotype increased: from 1.28 (95% CI 0.96 to 1.71) for studies with the smallest difference in homocysteine (bottom tertile) to 1.67 (95% CI 1.18 to 2.35) in studies with the largest difference in homocysteine (top tertile). However, there was substantial overlap in the confidence intervals by category and the metaregression analysis *p* value was 0.34. No trend was noted for heterozygous individuals (webappendix p 15).

When we included all studies, the coefficient for the Egger test was 1.15 (95% CI 0.65 to 1.64; *p* < 0.0001) for TT versus CC and 0.90 (95% CI 0.37 to 1.42) for CT versus CC (*p* = 0.001). Since this finding could be attributable to differences in the genotypic effect by folate categories (suggested in figure 1 and figure 3), we ran the Egger test in studies from each geographical region separately. The respective coefficients for the Egger test were (for TT vs CC comparisons) 1.20 (95% CI 0.48 to 1.92; *p* = 0.002) for Asia; 0.51 (95% CI –0.55 to 1.57; *p* = 0.318) for Europe, low; 1.08 (95% CI 0.02 to 2.15; *p* = 0.046) for Europe, mid; 2.91 (95% CI –2.42 to 8.24; *p* = 0.143) for America, Australia, and New Zealand, mid; and 1.29 (95% CI –1.59 to 4.17; *p* = 0.250) for America, Australia, and New Zealand, high.

Apart from being a signal in genome-wide association studies for homocysteine, we did not find reports of an association of the rs1801133 (*MTHFR* 677T) variant with established risk factors for stroke, or for any SNPs in linkage disequilibrium with it (defined as $r^2 > 0.3$) (webappendix p 23). Although one SNP in *MTHFR* (rs17367504) was identified as a genome-wide association study locus for systolic blood pressure, the linkage disequilibrium between rs17367504 and rs1801133 is low (r^2 0.09 European and Asian, 0.01 African ancestry; 1000 Genomes Project Pilot 1) and is unlikely to account for the association between rs1801133 and stroke.

13 randomised trials of homocysteine-lowering interventions including 45 549 individuals (2314 stroke events and 269 transient ischaemic attacks) met our selection criteria for analysis of *MTHFR* 677C→T and stroke in the context of randomised trials of homocysteine-lowering interventions (webappendix p 24). Trials identified included mainly participants with vascular disease (coronary heart disease or stroke), chronic kidney disease, or diabetes. We identified no trials exclusively set in low folate regions (Asia) that fulfilled our selection criteria. The main outcome for analysis consisted of both fatal and non-fatal ischaemic or haemorrhagic stroke and the weighted mean follow-up duration was 4.7 years (range 2–7.3 years).

Active treatment resulted in a mean reduction in homocysteine concentration of 3.33 $\mu\text{mol/L}$ (24% proportionate reduction) and a relative risk of stroke of 0.94 (95% CI 0.85 to 1.04; $I^2=18.8\%$; figure 4). Metaregression analyses of the log of the relative risk for stroke against the mean net or proportional reduction in homocysteine achieved by the intervention, or against the probable folate status category, did not show an association ($p>0.5$). With the probable exception of HOPE-2, no trial had an undue effect on the estimate of the pooled relative risk (webappendix p 16). A subgroup analysis did not reveal major differences of the intervention on stroke by prespecified study-level characteristic, with the possible exceptions of sample size and risk of bias (figure 5).

When we compared the effect from randomised trials undertaken in non-Asian populations to the effect of large genetic studies in the same population, a concordance of no reduction in risk of stroke was noted: relative risk reduction 6% (95% CI -4 to 15) and 0% (95% CI -11 to 10), respectively (figure 4). The relative risk reduction suggested by large Asian genetic studies of 22% (95% CI 10–32) could not be directly compared with interventional trials because no randomised trial has been done exclusively in these regions.

Discussion

The *MTHFR* 677C→T variant was associated with a larger effect on homocysteine concentration in regions of low folate consumption than in regions with high dietary folate intake or with established programmes of folic acid fortification of flour for prevention of neural tube defects. A similar pattern was noted for the genetic association with stroke risk. However, even though our analysis is less likely to be affected by small-study bias than was our previous meta-analysis, whether this difference reflects modification of the genetic association with stroke risk by population folate consumption or small-study bias remains unclear (panel).

Even if the evidence of an increased risk of stroke associated with the *MTHFR* 677C→T variant is robust, reduction of homocysteine concentration might not necessarily reverse the risk of stroke in adult life. Such evidence can only be derived from randomised trials of interventions such as folic acid that reduce the concentration of homocysteine. Our meta-analysis of randomised trials evaluating homocysteine-lowering treatments in stroke showed a non-significant reduction in the risk of stroke of 6% (95% CI -4 to 15). We note, however, that with the exception of half the participants from the VITATOPS trial who were recruited from low folate regions (Asia), the rest of the participants ($n=41\,467$, 91%) included in trials to date were from regions with high concentrations of folic acid or with established policies of folic acid fortification. The point estimate derived from the randomised trials was, however, concordant with that predicted from large genetic studies (≥ 400 stroke events) undertaken outside Asia (0% risk reduction; 95% CI -11 to 10; figure 4). The concordance of results, showing no benefit on stroke prevention from randomised trials and large genetic studies in non-Asian populations, suggests that homocysteine-lowering treatments in populations already fortified or with increasing population levels of folic acid might not provide additional benefit in reduction of the risk of stroke. Similar triangulation of genetic studies and randomised trials undertaken in low folate regions was hampered by the scarcity of evidence from randomised trials set in such regions (only 9% of all randomised trial participants). Nonetheless, the predicted risk reduction in stroke of 22% (95% CI 10 to 32) derived from large genetic studies in low folate regions suggests that if an adequately powered randomised trial were to be done in Asia, homocysteine reduction might have a substantial protective effect on stroke. However, our data also suggest that we cannot reliably exclude that the effect of the *MTHFR* 677C→T variant on stroke reported in large genetic studies done in low folate regions is attributable to other mechanisms different from lowering of homocysteine, or to small-study bias.

By contrast with our genetic meta-analyses, the estimate from randomised trials did not seem to be affected by the reduction in homocysteine achieved or by probable folate status of the population studied. However, this analysis was based on study-level characteristics, and thus the power to detect real differences with only 13 studies (as opposed to 101 genetic studies) was restricted, and the proportional reduction of homocysteine concentrations for most trials was fairly similar (median 26%, range 8–31), with the exception of one very small trial that achieved a 44% reduction. Nonetheless, a previous meta-analysis of homocysteine reduction and vascular events found no evidence of heterogeneity when stratifying by pretreatment or percentage reduction of plasma homocysteine or by regional folate fortification. Furthermore, the VITATOPS trial did not find evidence that individuals of Asian origin had a different effect from homocysteine reduction than did other individuals.

A few differences between the evidence obtained from randomised trials and genetic studies are important to emphasise. First, as already mentioned, most trial evidence arose from settings in which policies of folic acid fortification have already been implemented (ie, America, Australia, and New Zealand) or in which folate concentrations at the population level have been increasing in recent years. By contrast, around 40% of stroke events in the genetic studies that we analysed are from unfortified regions or populations with low concentrations of folate (Asia or Europe, low). Second, despite the extent of reduction in homocysteine concentrations being largely similar across the genetic studies and randomised trials (3.33 $\mu\text{mol/L}$ for trials and 2.58 $\mu\text{mol/L}$ for large genetic studies), the length of follow-up was substantially different. Randomised trials had a weighted average follow-up of 4.7 years and allocation to the intervention occurred in middle age (mean age range 56–69 years). By contrast, genetic studies reflect lifetime exposure to the phenotype resulting from the genetic variant, since randomisation to the allele variants occurs at conception. Third, the number of stroke events in trials is substantially lower than that included in the genetic analyses (2314 stroke events in trials vs 20 885 events in genetic studies), thus the meta-analysis of randomised trials had fairly low statistical power to detect small benefits or those noted mainly in a subgroup (eg, low folate regions). Fourth, evidence from randomised trials is derived mainly from individuals with established vascular disease, whereas that from genetic studies is mainly population-based. Therefore, genetic evidence should be regarded only as an approximate guide to the risk reduction that could be achieved by modification of homocysteine concentrations in randomised trials.

Although mendelian randomisation analysis reduces biases and confounding seen in non-genetic observational studies, it can still be prone to small-study bias and confounding by linkage disequilibrium. Our in-silico analysis evaluating the long-range linkage disequilibrium of the *MTHFR* 677C→T variant, used as an instrument for homocysteine, suggests that the differential genetic results by folate regions are unlikely to arise by differences in linkage disequilibrium between folate regions. To minimise the effect of small-study bias, we made great efforts to rescue data from unpublished studies and undertook, at the analytical stage, several sensitivity analyses that attempted to quantify the effect of small studies. First, restriction of the analysis to studies with at least 400 cases yielded an OR of 1.28 (95% CI 1.11 to 1.48; figure 3). Second, taking into account the number and potential outcome of missing data in a trim-and-fill analysis resulted in an adjusted summary OR of 1.30 (95% CI 1.19 to 1.43; webappendix p 14). Finally, using the observed distribution of *MTHFR* C/T genotypes, we estimated that 41 additional null studies with 400 cases and 400 controls each would be needed to reduce the overall OR from 1.68 to 1.15, the threshold OR at which cumulative evidence would be deemed questionable on the basis of Venice criteria (webappendix pp 4–5, 22). However, these analyses cannot wholly adjust for potential small-study bias and residual bias might persist, which

emphasises the need for verification of the findings in large genetic studies undertaken in low folate regions.

We used the *MTHFR* 677C→T variant as a genetic instrument to evaluate the effects of homocysteine on disease risk; however, the poor specificity of this variant (a situation inherent for trans variants used as instruments of non-protein traits) makes it possible that the genetic effect of *MTHFR* could be by a mechanism other than homocysteine. The use of probable folate status categories represents an estimate of population average with the possibility of substantial variability of natural folate intake within each category. This approach could underestimate the effect of folate status categories on homocysteine concentration and stroke risk. By contrast, the use of categories of probable folate status instead of folate concentrations from individual participants creates the possibility that the effect of the *MTHFR* variant by categories of population folate levels could be due to a strong association with another factor causally involved in the development of stroke. However, the plausibility of *MTHFR*–folic acid interaction is supported by the CARDIA study, which showed that the effect of the *MTHFR* variant on homocysteine concentration is modified by participant folate concentrations, and that such effect is largely diminished after the establishment of a policy of folic acid fortification. Finally, the effect seen in low folate regions could be due to selective reporting bias in the subtype of stroke reported in genetic case-control or cohort studies—ie, if stroke subtype was differentially reported according to continent and was associated with *MTHFR* genotype, the observed signal could be inflated. In an attempt to minimise this bias, we contacted authors of all studies and asked for full details of all stroke subtypes, including unpublished data. Furthermore, when we examined the subtypes of stroke contributing to our main stroke outcome, there was general consistency in stroke subtype by folate region, with the exception of America, Australia, and New Zealand, mid (in which all strokes included were ischaemic). However, we cannot exclude the presence of selective outcome reporting bias that could affect our results.

In conclusion, the concordance of findings on the genetic effects of *MTHFR* on homocysteine concentrations and the genetic association with stroke risk argues in favour of effect modification by prevailing folate concentrations in the population. If correct, this genetic finding would have important repercussions for the interpretation of clinical trials of homocysteine-lowering interventions. The effect of homocysteine-lowering interventions on stroke might be expected to be null in regions with high dietary folate intake or where food is fortified with folate, but it could be substantial in low folate regions such as Asia. Since stroke rates are high in Asian countries and the population at risk in Asia is very large, the public health benefit could be of great importance. Unfortunately, the evidence from randomised trials undertaken in low folate settings is scarce and genetic studies in the same settings have tended to be smaller than those in high folate settings, leaving a potential that estimates of the genetic effect have been inflated. To resolve uncertainty about whether the association of the *MTHFR* variant with stroke risk in Asian studies is a consequence of small-study bias, low folate consumption in that region, or both, a large genetic study of stroke in a low folate region would be needed. If further trials are to be done to evaluate the efficacy of homocysteine-lowering interventions for stroke prevention, these should be undertaken in regions of low folate consumption. The ongoing China Stroke Primary Prevention Trial (CSPT) is investigating 15 000 hypertensive individuals without established cardiovascular disease who are being randomly assigned to enalapril or enalapril plus folic acid and followed up for 5 years for development of the primary outcome, stroke. The addition of CSPT to the existing but small-scale evidence from randomised trials in low folate regions should yield a more definitive answer on the role (if any) of folic acid supplementation and lowering of homocysteine in prevention of stroke.

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Web Extra Material

Supplementary Material 1. **Supplementary webappendix.**

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pharmaceutical company, and in 2006, after completion and full analysis of ESPRIT, the study group accepted financial support from Boehringer Ingelheim for post-hoc exploratory analyses of the ESPRIT trial data. For this purpose a contract was signed in negotiated complete scientific freedom. ML has received money for board membership, consultancy, expert testimony, grants, and lectures from various pharmaceutical companies. JH has received consultancy fees from Merck Serono, Eisai, and Johnson & Johnson. BBW is co-principal investigator of the NIH funded GARNET, which funds genome-wide association studies of genetic samples from the Vitamin Intervention as Stroke Prevention randomised controlled trial. GH has received funds from Johnson & Johnson (executive committee for ROCKET-AF trial); Sanofi-Aventis (executive committee BOREALIS trial); Schering-Plough (steering committee TRA 2P TIMI 50 trial); Pradaxa (dabigatran) advisory board, Australia; and payment for lectures at sponsored scientific symposia by Sanofi-Aventis and Pfizer.

Panel

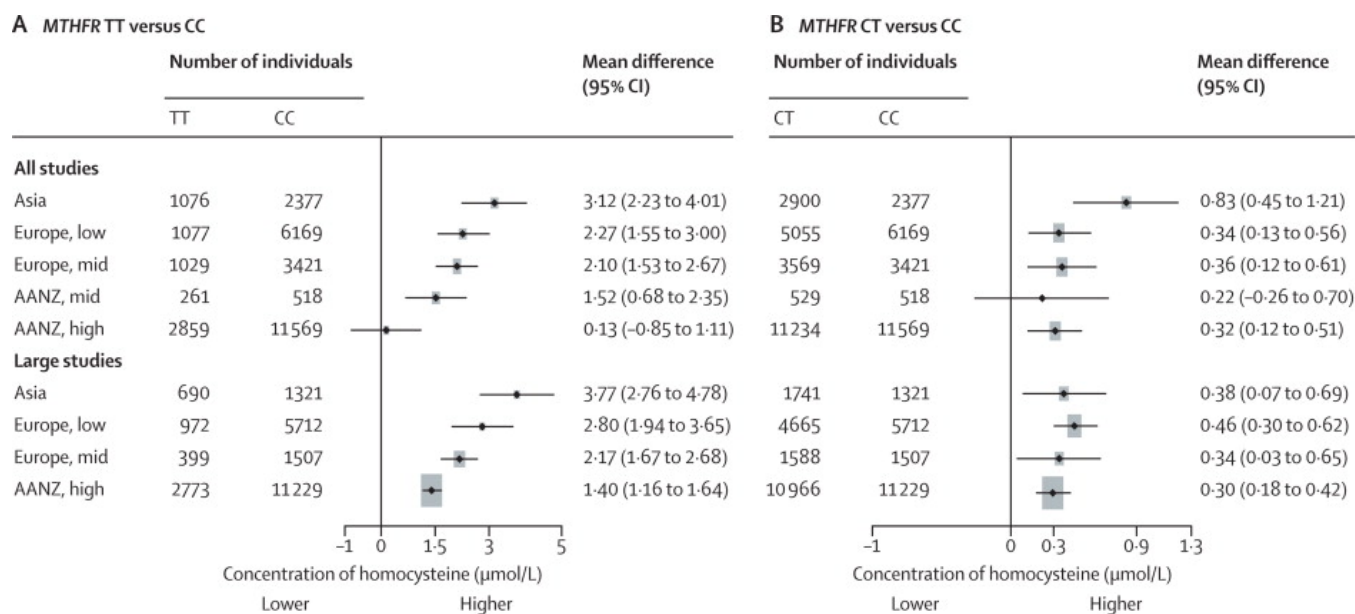
Research in context

Systematic review

Observational studies have shown an association between circulating concentrations of homocysteine and risk of stroke; however, these findings might arise from confounding or reverse causation, or both. Variants in *MTHFR* 677C→T (rs1801133), used as a non-confounded proxy of homocysteine exposure, have confirmed this association in previous meta-analysis; however, the magnitude of association differed by geographical location. This finding suggests that the association between homocysteine and stroke risk might be modified by prevailing folate intake, but another explanation could be small-study bias. Furthermore, findings from meta-analysis of randomised controlled trials investigating homocysteine-lowering interventions in stroke have been null.

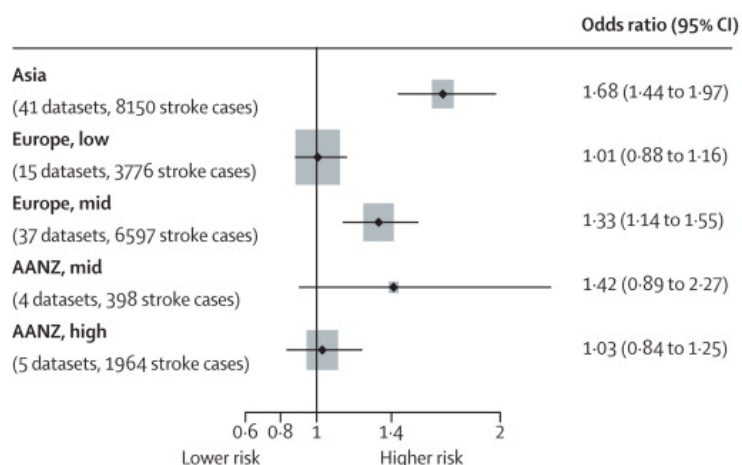
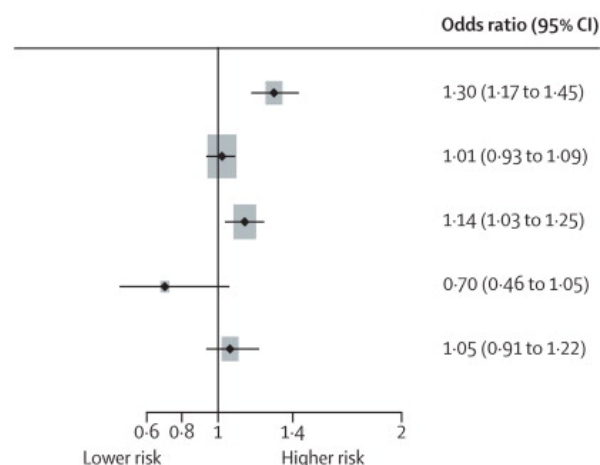
Interpretation

The effects of *MTHFR* 677C→T on homocysteine decreased as the prevailing level of folic acid increased. These effects were mirrored in the analysis of *MTHFR* 677C→T and stroke: an association between *MTHFR* 677C→T and stroke was only evident in a region associated with low levels of folate, findings that were preserved in large studies. In regions with increasing levels of folate or with established policies of folic acid supplementation, the effect of *MTHFR* 677C→T on stroke was null. These findings argue in favour of a gene–environment interaction. When we attempted to frame findings from *MTHFR* genetic studies within the context of homocysteine-lowering randomised trials evaluating risk of stroke, randomised trials were predominantly undertaken in regions with existing folic acid supplementation, which could potentially account for the null summary estimate of randomised trials, and hindered our comparison. Large genetic studies on stroke in regions with low folate levels are needed to confirm or refute our findings. If future trials of homocysteine-lowering interventions for stroke risk are to be undertaken, they should be set in low folate regions.

**Figure 1.**

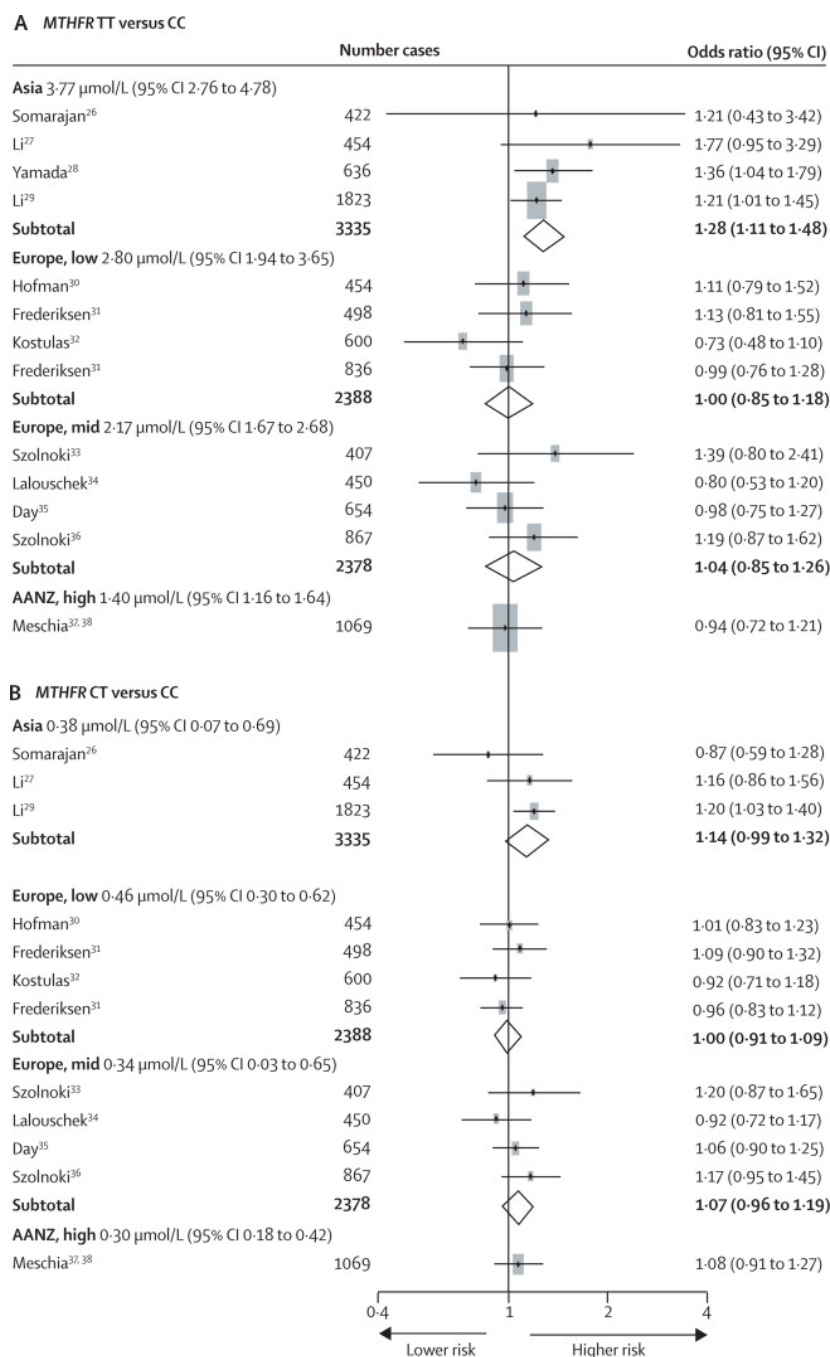
Difference in homocysteine concentration in individuals without cardiovascular disease according to *MTHFR* 677C→T genotype, by probable folate status category

Large studies are those with more than 500 individuals. AANZ=America, Australia, and New Zealand.

A *MTHFR* TT versus CC**B** *MTHFR* CT versus CC**Figure 2.**

Odds ratio of stroke according to *MTHFR* 677C→T variant, by probable folate status category

Left panel compares individuals homozygous for T allele with CC participants. Right panel compares heterozygous with CC individuals. AANZ=America, Australia, and New Zealand.

**Figure 3.**

Odds ratio of stroke in large studies (≥ 400 stroke events) according to *MTHFR* 677C→T genotype, by probable folate status category

Differences in homocysteine concentration (derived from large studies; figure 1) are shown in category subheadings.

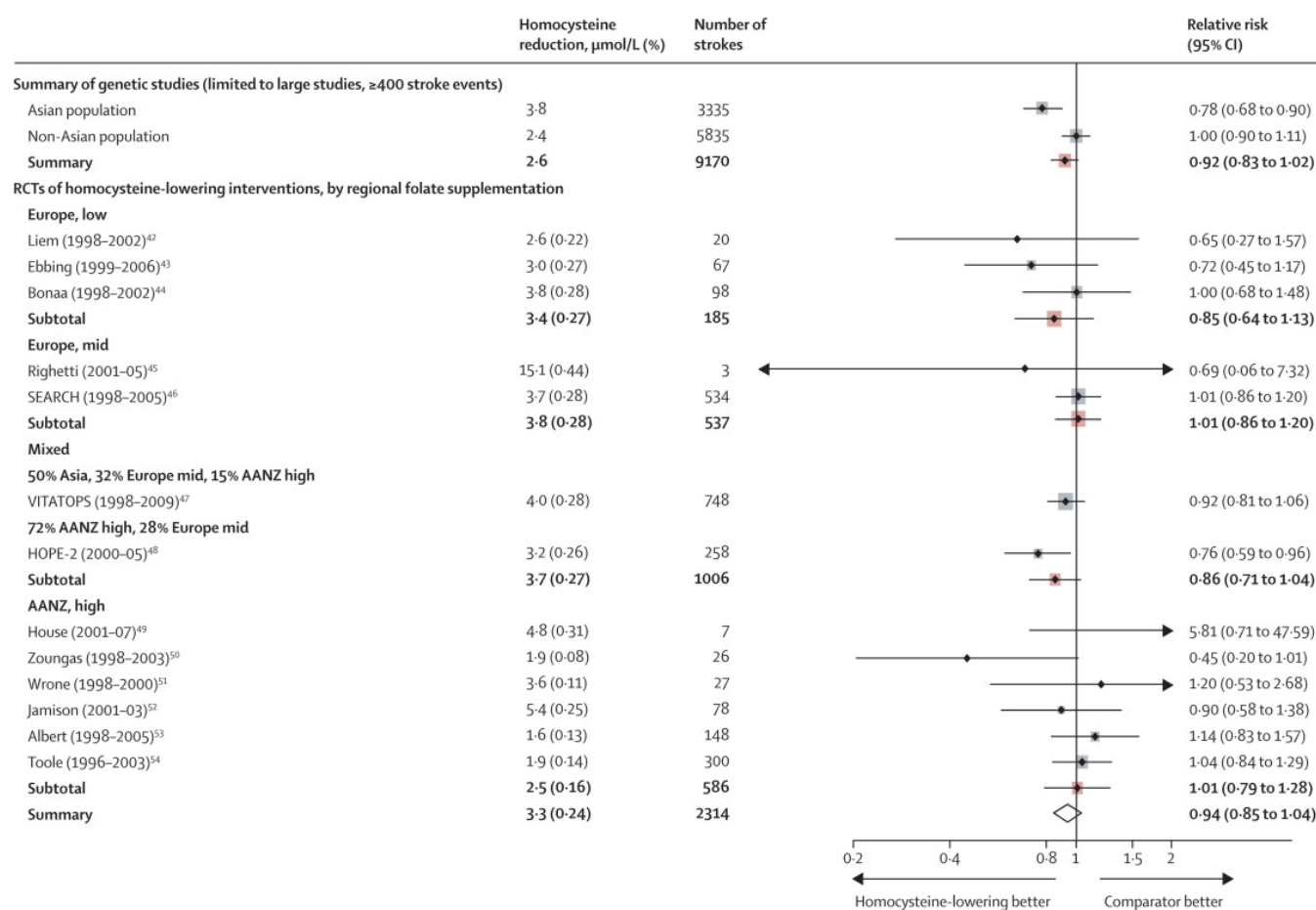
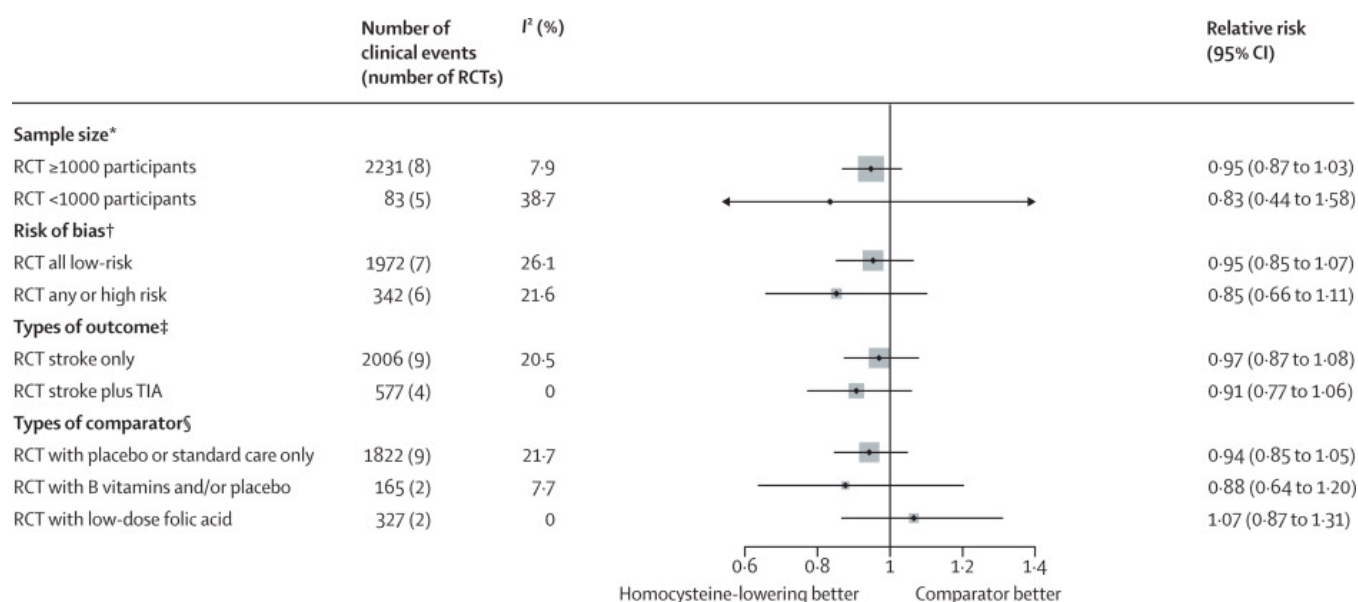


Figure 4.
Pooled relative risk of stroke from randomised clinical trials of homocysteine-lowering interventions in the context of genetic studies of the MTHFR 677C→T variant
RCT=randomised controlled trials. AANZ=America, Australia, and New Zealand.

**Figure 5.**

Sensitivity analyses of effect of homocysteine-lowering interventions from randomised clinical trials on risk of stroke

All analyses are random effects (DerSimonian and Laird). Number of clinical events reports stroke only as default and stroke plus TIA when specified in row title. RCT=randomised controlled trial. TIA=transient ischaemic attack. *Studies with fewer than 1000 participants were Wrone, Zoungas, Liem, Righetti, and House. †See webappendix p 24 for classification of risk of bias per study. ‡Studies reporting stroke and TIA separately were Righetti, Wrone, and HOPE-2. §Studies that used B vitamins (other than folic acid) as one of the comparator groups were Ebbing and Bonaa; studies that used low-dose folic acid as comparator group (and not placebo or standard care) were Wrone and Toole.